Increased Production of Sugar Streams from Lignocellulosic Materials through Enzyme Recycling

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By

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Abstract

The Canadian pulp and paper mills generate over 1 million tons of primary sludge per year. This sludge mainly contains small cellulosic fibers and contaminants from pulp and paper manufacture and is usually disposed of by landfilling or incineration. Both of these processes are economically and environmentally objectionable. However, the mill sludge could serve as low or negative-cost cellulosic feedstock that requires no pre-treatment and is largely available. The objective of this study was to examine primary sludge as a source of fermentable sugars that are used for production of biofuels and value-added biochemicals. Sludge was hydrolyzed with a commercial enzyme in presence of surfactants that are known to enhance the efficiency of enzymatic hydrolysis. The hydrolysis process was statistically optimized using response surface methodology. In addition, opportunities for enzyme recovery and reuse were also investigated. The primary sludge contained 30% of dry solids of which 51% was glucan. The study of different surfactants revealed that glucose yield from sludge can be improved by up to 12% in presence of polyethylene glycol 4000. Statistical model pointed the solid and enzyme loadings as the most significant factors (p < 0.05) in enzymatic hydrolysis of sludge. Under optimum conditions of 7.4% w/w dry solid loadings, 2.6% enzyme loadings (19.2 FPU g⁻¹ dry primary sludge, and 5% w/w surfactant loadings (polyethylene glycol 4000), an 85.6% glucose recovery of the theoretical maximum was attained. Furthermore, 34.7% enzyme was recovered from the sludge hydrolysate using a 3 kDa molecular weight cut off ultrafiltration membrane. The recovered enzyme was reused on fresh sludge sample to produce a glucose yield of 82.3%. Present work suggests that primary sludge can add value in the form of fermentable sugars. It presents an opportunity to reuse nearly 35% of the enzyme, which could significantly reduce the production costs of fermentable sugars. Thus, enzymatic
conversion of primary mill sludge can offer the pulp and paper industry an alternative approach for turning waste into value in a cost-efficient and environmentally friendly way.

**Keywords**

Pulp primary sludge; Enzymatic hydrolysis; Response surface methodology; Fermentable sugars; Enzyme recycling
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Abbreviations

PS, primary sludge;
CAD, Canadian Dollar;
CCME, Canadian Council of Ministers of the Environment;
PHB, polyhydroxybutyrate;
PHA, polyhydroxyalkanoate;
HPLC, high performance liquid chromatography;
PEG, polyethylene glycol;
FPU g⁻¹, filter paper unit per gram;
DPS, dry primary sludge;
kDa, kilo dalton;
RI, refractive index;
g, gram;
mL- milli liter;
µL, micro liter;
L- liter;
mM- milli molar;
h- hour;
ANOVA-analysis of variance;
R²- regression coefficient;
DF, degree of freedom;
MSW, municipal solid waste.
CHAPTER 1: LITERATURE REVIEW

1. Introduction

1.1. Global energy and challenges associated with fossil fuel

There are various energy sources currently being utilized globally. According to a report by World Energy Council (2013), the highest proportion of the global energy source is from fossil fuel, which by 2011 accounted for 82% of the global energy production. Other energy sources such as nuclear, hydro, and renewable energy source accounted for 5%, 2% and 11% of the total production respectively. There has been an increase in energy production from 9908 Million Tons of Oil Equivalent (Mtoe) in 1993 to 14 092 Mtoe in 2012 and is projected to reach 17 208 Mtoe by 2020. It is projected that fossil fuel will continue to dominate the global energy market (Levitan et al., 2014). However, the production and consumption of other forms of energy, especially the renewable energy source are indicated to slowly increase (Ellabban et al., 2014) as concerns over the impact of fossil fuel intensify (Janaun & Ellis, 2010).

The use of fossil fuel energy is associated with various challenges. One of the challenges is associated with the diminishing fossil fuel reserves (Román-Leshkov et al., 2007; Hartnady, 2010; Rahman et al., 2014). Owen et al., (2010) noted that the demand for increased supply of fossil to increased industrialization results in the depletion of the conventional sources of fossil fuels. The under production of fossil fuels is also associated with excessively high prices that result in the increase in the production costs of manufactured and processed products (Coady et al., 2010; Ji & Fan, 2012). (Panwar et al., 2011) argued that the use of fossil fuel leads to adverse effects on the climate. The increase in global warming and the associated effects such as flooding, droughts, and fluctuation in the global temperature is associated with the excessive burning of fossil fuel
These challenges have led to the need to explore other alternative sources of fuel.

1.2. Alternative energy sources

The solution to the challenges posed by the use of fossil fuel energy can be addressed through the use of an environmentally friendly and sustainable source of energy (Panwar et al., 2011; York, 2012). The alternative form of energy should be able to address the global energy needs, however, as indicated by the World Energy Council report (2013), phasing out of fossil fuel production will lead to energy supply gap. Therefore, reduction of the fossil fuel consumption is the most viable way of mitigating the environmental effect associated with its use (York, 2012).

Renewable energy sources present the best alternative to fossil fuel use (Panwar et al., 2011; York, 2012). Examples of renewable energy sources include solar energy, wind energy, biomass energy, geothermal energy (Evans et al., 2009). These energy sources do not produce environmental pollutants and can be reused, hence are sustainable (Edenhofe et al., 2011). The adoption of renewable energy also increases accessibility to energy in rural areas since it is cheaper and can be locally produced (Cook, 2011). However, as indicated above, the ability of the renewable energy sources to replace fossil fuel energy is based on the volume of production. According to Kralova and Sjöblom, (2010), biomass energy has the highest production volume (1313 Mtoe) compared to other forms of renewable energy such as solar energy (0.4 Mtoe), wind energy (44 Mtoe) and geothermal energy (86 Mtoe). Kralova and Sjöblom, (2010) projected that the production volume of biomass energy will be 3271 Mtoe by 2040, accounting for 24% of the global energy demand. Based on the present and projected biomass energy production (Kralova
& Sjöblom, 2010), this form of energy is the most suitable alternative energy source for fossil fuel use. Examples of biomass energy include the landfill gas and biogas, solid waste, wood and agricultural products, ethanol and biodiesel (Chandra et al., 2012; Demirbas et al., 2011).

1.3. Grain-derived fuels and associated challenges

Fuels such as ethanol can be developed from grains such as maize grains through the wet milling process or the dry milling option. In both processes, fermentation is done to convert the products to alcohol (Mojović et al., 2006; Talebnia et al., 2010). In the wet milling process, the grains are prepared by separating the kernel into different parts such as germ, fiber, and starch. However, for the dry milling process, the whole grain kernel is milled to flour. Milling is important in creating larger surfaces area for water contact and for enzyme-controlled degradation of starch. After the milling stage, the starch contained in the grains is gelatinized through the addition of water and enzymes in slurry tanks. The resulting slurry is then sorted through the removal of the solid components, which are returned to the milling stage and back to the system. The liquid part of the gelatinized slurry is then taken to the primary liquefaction stage where the enzymes break down the starch to glucose (Mojović et al., 2006). The produced mash containing glucose then undergoes fermentation, which involves the conversion of simple sugars to ethanol and carbon dioxide. The fermentation process is enabled by the addition of yeast (Solomon et al., 2007; Talebnia et al., 2010). The fermentation product is then distilled to enable achieve the separation of the grain-derived bioethanol fuel based on the unique boiling points of the ethanol. The produced ethanol is further subjected to molecular sieving to concentrate and purifying the biofuel by selective trapping of water molecules resulting in a highly pure ethanol product (Mojović et al., 2006; Solomon et al., 2007). Other additional steps to further develop the product include
centrifugation and evaporation. From the process above, it is evident that the production of glucose from the grains is an important step. The amount of glucose produced determines the quantity of biofuel produced.

The advantages associated with the production of ethanol from grains such as maize grain include its cost effectiveness (McAlloon et al., 2000), which have resulted in the increased expansion of land under grain crops grown for bioethanol production in countries such as the US (Cotula et al., 2008; Westhoff, & Brown, 2008). It is indicated that in 2010 about 30% of the land under maize production in the US was dedicated to bioethanol production up from 13% in 2005 (Westhoff, & Brown, 2008). The use of food crops is also indicated to contribute to higher economic growth. However, the production of grain-derived fuels has been met by opposition from individuals and organization arguing that the approach leads to food shortage (Rathmann et al., 2010; Ajanovic, 2011; Chen & Khanna, 2012). In a world that is faced with the challenge of food security, the use of food grains to produce bioethanol at the expense of feeding the populations dying of hunger such as those in impoverished nations of Africa is regarded as insensitive and inhumane (Ajanovic, 2011; Chen & Khanna, 2012). The food versus fuel conflict has led to a slowdown in the production of grain-derived fuels and intensified the need for an alternative source of biomass energy (Karp & Richter, 2011). Other sources of glucose such as lignocellulose should, therefore, be considered (Li et al., 2012; Schmitt et al., 2012).
1.4. Use of lignocellulose biomass in energy production

1.4.1. Source of lignocellulose biomass

Lignocellulose biomass is a term that is used to describe the non-seed plant biomass (Hadar, 2013). Lignocelluloses are widely distributed and make up the highest percentage of biomass (Balat, 2011). The sources of lignocellulose biomass include agricultural plant products such as wheat straws and other non-edible plants parts discarded after harvesting. Some non-edible crops such as jatropha, pongamia, madhuca and azadirachta are specifically grown to be used as sources of lignocellulose (Mathiyazhagan et al., 2011). Lignocellulose can also be obtained from agro-industrial sources such as municipal solid wastes (Li et al., 2012; Schmitt et al., 2012). Forest products such as fast growing trees can also be used as the source of biomass (Seguim 2011). Forest products account for close to 370 million tons of lignocellulose produced and used in the US (Hadar, 2013). However, the scarcity of forest and the importance of forest in carbon dioxide regulation limit its use as a sustainable source of lignocellulose biomass (Balat, 2011; Hadar, 2013).

1.4.2. Benefits and problems of lignocellulose-derived fuels

The production of biofuels using lignocellulose biomass is advantageous since the plant parts are known to have a high concentration of renewable energy resources (Hadar, 2013). The use of lignocellulose from non-edible plant parts and plants such as jatropha also helps to resolve the food versus fuel conflict that is experienced with the use of edible food (Kullander, 2010; Valentine et al., 2012), hence the lignocellulose-derived fuels are more sustainable over the long term (Hadar, 2013).
However, there are challenges that are faced when using lignocellulose as feedstock in the production of fuel. One of them is the high cost of production (Mathiyazhagan et al., 2011). The production of commercially grown plants for use in biofuel production is associated with high agricultural cost due to high water, fertilizers and labor demand (Mathiyazhagan et al., 2011). However, the plant production cost can be reduced through the use of plants such as Jatropha and selected weeds, which require lower agricultural inputs (Mofijur et al., 2012). The production of lignocellulose-derived fuels is also affected by its high energy demand (Balat, 2011). The harsh conditions through which glucose is generated from the native structure of lignocellulose biomass makes the use of the biomass to complicated and potentially costly (Hadar, 2013).

1.4.3. Structure and composition of biomass

As indicate by Hadar (2013), the molecular structure of the lignocellulose biomass is the main hindrance to its use. However, it should be noted that lignocelluloses are packed with a high concentration of glucose (Zhao et al., 2012), therefore, understanding its structure is important in the development of strategies to enable the release of the glucose from the biomass.

Arrangement of various components within the lignocellulosic fiber matrix

(Mood et al., 2013)
Lignocellulose biomass is mainly composed of cellulose, hemicellulose, and lignin (Zhao et al., 2012; Monlau et al., 2013). The three components are tightly packed together. The cellulose molecules make up to 50% of the biomass and are located in the inner part of the biomass. The cellulose molecules are made up of continuous chains of glucose monomers linked together by a β-1,4 glycosidic bond. Several molecules of cellulose polymers found in lignocelluloses biomass are aggregated through hydrogen bonding forming cellulose microfibrils (Zhao et al., 2012; Monlau et al., 2013). The resulting hydrogen bonding causes the cellulose to assume a crystalline structure that covers two-thirds of the cellulose microfibrils while the rest of the structure is amorphous. The crystalline structure of cellulose makes the molecule to be insoluble and reduces its degradability (Glasser et al., 2012).

Another component of lignocellulose is the hemicellulose, which unlike cellulose is made up of different types of sugars and contains additional molecules such as glucuronic, and ferulic acids. The sugars that make up hemicellulose include L-arabinose, D-galactose, D-glucose, D-mannose, and D-xylose. Unlike cellulose, the hemicellulose is made up of both branched and linear chains. There are different types of hemicellulose, classified based on the dominant sugars, examples include the xylans and glucans (Zhao et al., 2012). The fact that hemicelluloses are branched and contain an amorphous structure makes them more degradable compared to cellulose microfibrils. Hemicellulose makes up between 20-35% of the biomass.

Lignin is located at the outer section of the lignocellulose fibers and makes up to 25% of the biomass. The molecule glues together the components of the biomass (Monlau et al., 2013). The molecule is complex and is made up of phenylpropane monomer units that are joined together in a 3D structure (Halil et al., 2007). The phenylpropane monomers that make up the lignin molecule include p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. The structure of lignin
makes it impervious to water and also highly resistant to chemical hydrolysis and enzymatic breakdown. The molecule protects the other components of the biomass against microbial degradation (Zhao et al., 2012).

1.4.4. Development of lignocellulose-derived fuels and associated challenges

The release of sugars from lignocellulose biomass is a difficult process involving a series of harsh conditions (Zhao et al., 2012; Monlau et al., 2013). The complex structure of lignocellulose is responsible for the difficulty in obtaining sugar molecules from it (Monlau et al., 2013). The outer lignin material, which is impervious to water, resistant to chemical and enzymatic breakdown and microbial degradation makes it hard to access the glucose rich cellulose material. The presence of crystalline structure in the cellulose microfibrils further makes the cellulose to be less susceptible to enzyme hydrolysis. Therefore, the combination of the outer lignin molecule and the crystallinity of cellulose microfibrils complicates the breakdown of lignocellulose to simple sugars (Monlau et al., 2013).

Given the structure of lignocellulose biomass, the pretreatment steps to facilitate the release of glucose constitute the first step in the development of lignocellulose-derived fuels (Monlau et al., 2013). The pretreatments options include the physical pretreatment such as milling, physico-chemical that include stem explosion and wet oxidation, chemical pretreatment such as the use of alkali and organic solvents and fourth option is the biological pretreatments (Halil et al., 2007). The goal of the pretreatment stages is to disrupt the native structure of lignocellulose biomass and increase its permeability to enzymes. Pretreatment process also partially depolymerizes the cellulose and increases its degradability (Monlau et al., 2013).
The next step in the process is the enzymatic hydrolysis of the depolymerized cellulose to simple sugars such as glucose. The hemicelluloses are also broken down to pentose sugars such as the xyloses. The enzymatic hydrolysis is carried out by the cellulases and hemicellulases. The production process also involves the detoxification steps to remove the inhibitors such as furan and phenolic compounds before the fermentation process. The resulting simple sugars are then metabolized to ethanol through fermentation processes. The last step in the production of lignocellulose-derived fuels is the separation and purification of the fermentation products. This step involves the use of calcium lactate, carbon, and sulfuric acid. It is important to carefully select the separation and purification to limit the energy demand while ensuring the production of the high quality end product (Talebnia et al., 2010; Humbird et al., 2011; Menon & Rao, 2012).

1.4.5. Economic analysis for developing lignocellulose-derived fuels

The production of the lignocellulose-derived fuels can be hampered by the high cost. One of the drivers of the high cost is the feedstock that contributes to 40% of the cost. However, this cost can be reduced by the use of cost-effective feedstock such as organic wastes, selective weeds such as Lantana camara and municipal solid waste. The cost of the pretreatment steps also contributes the overall high cost. The use of hot water pretreatment option is associated with low cost ($0.81/L of ethanol) compared to use of dilute alkali, which is associated with a cost of $0.88/L of ethanol (Kumar & Murthy, 2011). The high product cost is also associated with the hydrolytic enzymes used. The cellulase and hemicellulose are expensive with estimated cost of 5.28 US dollars per cubic meter of ethanol produced. The high cost of the enzymes is associated with the large volume that is required in the production process (McAloon et al., 2000; Klein-Marcuschamer et al., 2012). Establishing ways to recycle the enzymes can greatly reduce the cost of production. The cost associated with the use of the enzymes can also be cut by the use of low-
cost, effective enzymes such as cellulolytic enzyme from saccharolytic microorganisms. The cost of production of lignocellulose-derived fuels can also be scaled down through the adoption of large integrated production processes over the small ones. It is indicated that a 10-fold increase in the size of the production unit results in up to 50% reduction in the production cost (Bindraban et al., 2009).

1.5. Lignocellulose hydrolytic enzymes

1.5.1. Enzyme structure

The characteristic of the enzymes involved in the hydrolysis of the lignocellulose biomass depends on the producing organisms. The enzymes are mainly composed of features such as catalytic domain, cellulose binding domains, fibronectin-type III domain and NodB-like domain. Hydrolytic enzymes produced by aerobic microbes contain catalytic domains that are linked with the cellulose binding domains, which play an important role in the binding of cellulose crystalline and amorphous structure. However, the catalytic domain in anaerobic organisms is linked with the dockerin domain (Bayer et al., 1998).

The structure of the enzymes is also dependent on the type of enzymes with the 3-D structure of endoglucanases being different from that of exoglucanases. The 3-D structure of the endoglucanase enzymes produced by Clostridium thermocellum is characterized by a (α/β) barrel with 2 β-bulges located at 3rd and the 7th strands. The carboxyl end of the barrel contains an acidic cleft in which the active site is situated. A highly folded subdomain made up of α-helices and 2 β-structure strands of amino acids form a binding cleft that extends from the vicinity of the active site to the top of the barrel. For Trichoderma reesei cellbiohydrolase CbhII exoglucanases, the
α/β barrel is made up 7 strands associated in α-helix conformation excluding the sixth and seventh irregularly organized strands. The carboxyl end of the barrel has an enclosed tunnel that is formed by two extensive loops. The tunnel contains the binding site and acts as a site for threading of the non-reducing end of the cellulose polymer (Li and Papageorgiou, 2011).

1.5.2. Mechanism of action of the hydrolytic enzymes

Biomass hydrolysis by the catalytic enzymes occurs through either the retaining mechanisms or the inverting mechanism. The exoglucanases, which breakdown the cellulose through the processive removal of the sugar molecules from one end of the chain usually uses the retaining mechanism (Knott et al., 2013). The mechanism occurs in two steps that involve the double removal of the residues, the first displacement step is coordinated by the acid group that donates a proton to the glycosidic oxygen resulting in hydrolysis of the bond to form 2 fragments; one with a non-reducing end and the other is glycosyl-enzyme intermediate. The intermediate then undergoes the second displacement reaction resulting in the maintenance of the initial configuration at anomeric carbon level (Knott et al., 2013).

The endoglucanases hydrolyze the biomass by randomly attaching to any position on the biomass chain and subsequently cleaving the chains to smaller units. These enzymes utilize the inverting mechanism in which the glycosidic oxygen is protonated as the hydroxyl ions are formed through the ionization of a water molecule by a negatively charged aspartate-201 residue. The hydroxyl ions then cause the bond cleavage by targeting the anomeric carbon and subsequent inversion of the configuration. This mechanism does not proceed via glycosyl-enzyme intermediate but instead occurs through oxocarbonium ion-like transition state. It is important to note that the hydrolytic enzymes coordinate and they are synchronized during biomass degradation. Each of the enzymes is equipped to carrying out different hydrolytic functions that
lead to the efficient breakdown of biomass. The exoglucanases and endoglucanases break down
the biomass to short chains called cellobiose and cellodextrins, which are then acted upon, by the
other group of hydrolytic enzymes called β-glucosidases and cellodextrinases to produce simple
sugars (Zverlov & Schwarz, 2008; Barker et al., 2010; Gonçalves et al., 2012).

1.6. Hydrolytic enzyme recycling

1.6.1. Methods of enzyme recycling

To solve the high cost of lignocellulose derived-fuel production, there is a need to reduce
the quantity of hydrolytic enzymes required, since the process is indicated to consume large
amounts of the enzyme. Some of the options that have been used to mitigate the cost associated
with high enzyme demand include enhanced hydrolysis yield through the adoption of enhanced
reactor configurations (Qi et al., 2011). The cost effectiveness of these approaches is yet to be
assessed. Enzymatic recycling, however, provides the most viable way of managing the quantity
of enzymes required in the production process (Shang et al. 2014). The other benefits of enzyme
recycling include increased enzyme conversion efficiency through enhanced enzyme-substrate
interaction time.

The enzymes can be found in the liquid fraction or bound to the solid substances at the end
of the production process. For the enzymes suspended in the liquid fraction, enzyme recycling can
be achieved through ultrafiltration of the supernatant (Chen et al. 2013). (Rodrigues et al 2014) or
through reabsorption of free cellulases on fresh substrates (Eckard et al. 2013; Ouyang et al. 2013;
Ultrafiltration of enzymes from the supernatant aims at separating the solid residues containing the cellulases (Qi et al., 2012). A membrane with the ability to allow the passage of all other contents of the solid residues except the hydrolytic enzymes is then used in the ultrafiltration process. The preferred cutoff for the membrane is 10 kDa (Yang et al., 2010). The retained enzymes are then reused in the production process. The second method is the reabsorption of free cellulases on the fresh substrate. This approach involves the exposure of the used enzymes contained in the hydrolysis mixture to the fresh substrate (Ouyang et al. 2013). This approach is based on the principle that cellulase enzymes have a high capacity to adsorb the introduced solid substrate. It should be noted that β-glucosidases have low adsorption capacity (Ouyang et al. 2013), hence recycling by reabsorption is limited. To recover the enzymes from the suspension, fresh substrates are added at concentrations equal to the first round of production, so as to ensure maximum adsorption sites for the enzymes. The mixture is then agitated for 2 hours after which the suspension is filtered or centrifuged to obtain the introduced substrate containing bound enzymes. The approach is reported to result in 88% enzyme recovery rate (Tu et al. 2007). The significant difference between the two methods is the inability of the reabsorption method to facilitate the recovery of β-glucosidase due to its low adsorption into the solid substrate (Tu et al. 2007; Ouyang et al. 2013).

The recycling of enzymes bound to solid substances at the end of the production process is more complex (Rodrigues et al. 2012) and involves the dissociation of the enzyme from the solid substance after which the enzymes are recovered. It is important to dissociate the enzymes from the solid substances of the previous production cycle since the direct use of the enzyme bound to these solids leads to a possible build-up of substances such as lignin. To facilitate the dissociation of the enzyme from these solids another substrate such as residual cellulose with a higher enzyme
affinity is used. The enzyme binds the introduced cellulose through cellulose-binding domains (Qi et al., 2011; Rodrigues et al. 2012). Lignin can also be used to recovery the enzymes from bound solids. Once detached from the bound solids the cellulase can then be recovered from either the cellulose or lignin through the alteration of the pH (Zhu et al., 2009; Du et al., 2012) or addition of chemicals (Tu et al., 2009).

1.6.2. Composition of current commercial enzymes

The current commercial enzymes formulation is aimed at achieving enzymes with desirable characteristics such as high catalytic efficiencies through enhanced catalytic breakdown of crystalline cellulose (Gusakov, 2011). The enzymes are also designed to achieve high thermostability to facilitate their action under high-temperature conditions. The commercial enzymes are also developed to increase their specific activity so as to achieve a high degree of hydrolysis with low quantity of the enzymes. The enzymes are also designed to resist the inhibition by the end products of hydrolysis such as cellulobiose and glucose and to enhance their shear resistances to enable them to withstand agitation during production and recycling process (Samaniuk et al., 2011). To achieve the above-stated characteristics, the commercial cellulase enzymes mixtures are made up of different types of enzymes. The enzymes range from simple cellulases to complex enzymes capable of breaking down the crystalline molecules. The current commercially available cellulase enzymes include the Accelerase®1500, Accelerase®XP, Accelerase®XC and Accelerase®BG produced by Genencor and cellic Ctec, and cellic Htec. Cellic CTeC produced by Novozymes (Verardi et al., 2012).

As indicated above, the current types of enzymes are made up of a cocktail of several cellulase enzymes (Horn et al., 2012). Accelerase®1500, for example is made up of a complex of exoglucanases, endoglucanases, hemicellulose and beta-glucosidase. Cellic HTec is also made up
of a mixture of several enzymes capable of degrading pretreated biomass to simple sugars (Pakarinen et al. 2014). The Accellerase® Duet generated by genetically engineered T. reesei is indicated to contain xylanases, beta-glucosidases, and endoglucanases. Some of the commercial enzymes are however made up of single type of enzyme such as Accellerase® BG, which is made up of only beta-glucosidases (Gusakov, 2011).

It is evident that the commercial preparations of enzymes contain more than a single cellulase enzyme. The use of a cocktail of cellulase enzymes enhances the hydrolytic process, however, the challenge with the current combination is in the recycling of the enzymes. The difficulty in recycling the enzymes is due to the fact that each of the enzymes has different behavior during production and recycling process. The combination of enzymes with poor adsorption into solid substances with those with high adsorption capacity provides a challenge regarding to the type of recycling process to be adopted (Haven & Jørgensen, 2013). This study revealed that β-glucosidase in the different commercial enzymes behave differently during the recovery process with the Cellic® CTec2 β-glucosidase adsorbing strongly to lignin while the one in Novozym 188 does not. However, the researchers indicated that the extent of adsorption of CTec2 β-glucosidase varied with the type of biomass and the type of lignocellulose pretreatment approach adopted. According to Haven and Jørgensen, (2013), the adsorption of CTec2 β-glucosidase to lignin complicates the process of enzyme recycling.

1.6.3. Current development in enzyme recycling for biomass hydrolysis

As observed in the previous section, the strong adsorption of cellulases to lignin, and cellulose (Rodrigues et al. 2012) complicates the process of enzyme recycling. Various approaches towards enhancing the desorption of the enzymes from such solids have been advanced. One of the approaches is the minimization of the available lignin concentration through the use of
pretreatment stages. Sipponen et al. (2014) observed that the surface area of lignin is significantly reduced by acid-catalysed hydrolysis of lignin-carbohydrate. These researchers also indicated that the reduction of the surface of lignin leads to the enhanced digestibility of the biomass, as the enzymes are less bound to lignin. The other current development involves the control of the adsorption and the desorption behavior of the enzymes (Nozaki et al., 2011).

One of the current approaches to controlling the adsorption and the desorption behavior of the enzymes is through the use of surfactants. It is indicated that the addition of Tween 80 increases the desorption of cellulase enzyme by about 67% (Tu et al., 2009). The use of Tween 80 has been shown to result in a high proportion of free enzyme in the production of ethanol in steam exploded Lodgepole pine (Tu et al., 2007). Increase in the recycling efficiency by more than 50% has been reported with the use of other detergents such as Triton X-100 using ethanol pretreated Lodgepole pine (Tu et al., 2007). The role of detergents in enhancing the recycling efficiency is based on the fact that they outcompete the cellulase for lignin binding sites (Eriksson et al., 2002). Other studies have shown that the use of polyhydric alcohols leads to higher cellulase recycling efficiency compared to the use of surfactants. Increased recovery of the enzymes bound to corn stover to about 76% was observed when ethylene glycol was added to the hydrolysis mixture (Qing et al., 2010). In a study carried out by Sipos et al., (2010), the addition of polyethylene glycol to the hydrolysis mixture resulted in the enhanced recovery of the action of the cellulase enzyme.

Recent developments towards enhanced recycling of cellulase enzyme have pointed to the possible use of proteins in the control of adsorption and the desorption behavior of the enzymes. The recently used proteins are the casein micelle, which are spherical shaped casein molecules complexes capable of remaining suspended in liquid substances (Eckard et al., 2013). The study by Eckard et al. (2013) suggested that the casein micelles are capable of blocking lignin molecules
in hydrolysis mixture, hence preventing the adsorption of the cellulase enzyme to the molecule and subsequently enhancing the recycling process.

The desorption of enzymes can also be enhanced through the control of the thermodynamic equilibrium position, this can be achieved by raising the temperature of the hydrolysis mixture to about 45 °C (Tu et al., 2009; Shang et al. 2014). The regulation of the pH is also indicated to be key in the control of the adsorption and the desorption behavior of the enzymes (Zhu et al., 2009; Du et al., 2012; Shang et al., 2014). An increase in the desorption by 94% was reported in a study that involved a shift in the pH from 8 to 13 (Zhu et al., 2009). The study by Shang et al. (2014) showed that an increase in pH from 4.8 to 10 results in an increase in enzyme recycling efficiency from 20% to 85%. It is, therefore, evident that increase in pH is important in enhancing enzyme recycling.

1.6.4. Current problems in enzyme recycling

The use of pH and temperature control in the enhancement of the enzyme recycling efficiency is indicated to be associated with compromised enzyme activity. The increase in temperature is indicated to enhance the enzyme recycling efficiencies, however, the temperature above 50 °C leads to decline in the enzyme activity and subsequent reduction in the production of biofuels (Lindedam et al., 2013; Calderon et al., 2014; Pakarinen et al., 2014; Rodrigues et al., 2014). It is, however, suggested that the exposure of the enzyme to a high temperature for a short period of time reduces the chances of enzyme inactivation (Pakarinen et al., 2014). The negative effect of high temperature (above 50 °C) on enzyme activity has been reported even with current commercial cellulase enzymes such as Cel7A (Rodrigues et al., 2012) and Novozym 188 (Calderon et al., 2014).
It is evident that alkaline pH enhances the cellulase recycling efficiency, however, the alkaline pH is also indicated to result in the decline in enzyme activity (McIntosh & Vancov, 2011; Rodrigues et al., 2012). The alkaline pH is indicated to alter the activity of the enzyme by causing a conformational change in the structure of the enzymes (Rodrigues et al., 2012). The use of surfactants has been indicated to improve the recovery of the cellulase enzyme, however, the build up of the surfactants in the hydrolysis media has been indicated to cause inhibition. This is especially the case when the surfactants are continuously added through the various production cycles (Xue et al., 2011). It is also indicated that the build up of sugars due to enhanced enzyme recycling due to the addition of surfactant results in enzyme inhibition (Xue et al., 2011).

1.6.5. Possible solutions to enzyme recycling challenges

The challenge associated with the build up in the concentration of surfactant in the hydrolysis media to the level that causes inhibition can be resolved through the introduction of washing stages at regular intervals. Xue et al. (2011) observed that the use of NaAc-HAc wash buffer at 30 mg/g enzyme results in enhanced enzyme efficiency. Xue et al. (2011) also propose that the use of washing stages during enzyme recycling helps to minimize the end-product (glucose) inhibition. Glucose inhibition can also be resolved by developing a production system whereby the saccharification and fermentation occur simultaneously (Drissen et al., 2009), hence ensuring that there is no build up of glucose.

The challenge associated with the enzyme denaturation at alkaline conditions can be solved by using an alkaline tolerant enzyme or by bioprospecting for microbe capable of producing cellulase enzyme that is tolerant to alkaline conditions. This approach can also be used to resolve the problem faced with the use of temperature regulation in the enzyme recycling. Various thermostable and alkaline tolerant cellulase enzyme have been found (Annamalai et al., 2011; Liu
et al., 2013). It is important to assess how the enzyme recycling conditions that include the addition of a surfactant, washing cycles and the use of optimum temperature and pH can be optimized to yield high recycling rates while limiting the enzyme inhibition. Optimization can be carried out using Response surface methodology (Tu et al., 2009). It is also important to further assess the application of casein micelles since they have not been shown to cause any inhibition or enzyme inactivation, yet they have been shown to increase enzyme recycling potential (Eckard et al., 2013).

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CHAPTER 2: OPTIMIZATION OF ENZYMATIC HYDROLYYSIS OF PRIMARY SLUDGE FOR GLUCOSE RECOVERY

2.1. Abstract

Sludge disposal is an expensive proposition which involves collection, de-watering and disposal. Biological conversion of fibrous sludge to energy serves the dual purpose of reducing sludge disposal costs and generating energy. High cost of enzymes is the main limiting factor which makes the overall process of conversion of cellulose to sugars. The current study evaluates the impact of different factors of enzymatic hydrolysis on enzymatic saccharification of primary sludge (PS) to glucose using a commercial enzyme preparation Cellic CTec2. The compositional analysis of PS showed 70% moisture and remaining 30% solids which contributed 61.6% polysaccharides (cellulose and hemicellulose), 11% lignin, 23.5% miscellaneous (proteins & extractives) and 3.9% ash. Surfactant was found to be a lignin blocker which increase the surface area for enzymatic hydrolysis of cellulosic materials. Non-ionic surfactant PEG-4000 showed maximum 43.9% glucose recovery which was 12% accelerated when compared to control. Response surface methodological optimization and model validation of enzymatic hydrolysis of PS showed maximum glucose recoveries of 85.6% with 7.4% solid loadings, 2.6% enzyme loadings (19.2 FPU/g solids), 5% PEG-4000 and hydrolysis time of 100h. Thus, present study showed effective enzymatic hydrolysis parameters optimization of PS for production of sugars which could be further explore for either biofuel or biochemical production.

Keywords: Enzymatic hydrolysis, CTec2, Primary sludge, Glucose recovery, Response surface methodology
2.2. Introduction

Lignocellulosic materials such as agricultural (sugar cane bagasse) and forest residues (saw dust), municipal solid waste (waste paper) woody crops and herbaceous (switch grass) materials in large quantities are available in many countries with various climatic conditions, making them suitable and potentially cheap feedstock for sustainable production of fuel ethanol. Such materials are abundant and competitive in price with petroleum, and cellulosic biomass can provide a sustainable resource that is truly unique for making organic products (Lynd et al., 1991). In addition such materials provide a new pathway to manufacture organic fuels and chemicals which can be useful in part of world where there is a shortage of petroleum resources. However, the majority of the total carbohydrates in biomass are presented in forms of lignocelluloses like cellulose, hemicellulose or lignin.

Carbohydrates are an important natural and renewable source most commonly available from lignocellulose biomass which further hydrolyzed into fermentative sugars using various biochemical or thermochemical processes into various value added products. These products include various value added biotechnological products such as fuels, biochemicals, biopolymers (Anwar et al., 2014; Isikgor and Becer, 2015). The most of these carbohydrate rich substrates are of food origin and its their utilization may create food problems. So it’s challenging to find out carbohydrate rich waste substrate is a promising source for bioenergy which will avoid food security (Sassi and Galarza, 2016). One good and abundant source of cellulosic biomass is the pulp mill primary sludge (PS) which is a waste from wood pulp processing industries. Pulp and paper mills generate 2.6 million tonnes of mixed sludge produced from primary and secondary treatment of wastes derived from wood sources, recycled paper products, and non-wood fibers every year (Elliott and Mahmood, 2005; Camberato et al., 2006). This sludge can be very useful resource for
fermentable sugar production as it is composed of 45-50% glucan and 10-14% xylan (Kang et al., 2010). The advantage of this sludge is that it is homogeneous, abundant and available at no cost. Most commonly used disposal method was land filling was became more frequently practiced as an outlet for sludge utilization. Some pulp manufacturing companies pay CA$ 300 ton\(^{-1}\) for sludge handling and CA$20-50 ton\(^{-1}\) dry sludge for land filling. Recently, the Canadian Council of Ministers of the Environment (CCME) declared some regulatory guidelines for land filling of pulp and paper sludge in Canada. Hence such disposal methods however are not economically and environmentally attractive.

Waste sludge hydrolysis into sugars may be an attractive option. Hydrolysis can be done by acid, alkali and/or enzymatic methods. Acid or alkali hydrolysis has some disadvantages such as product separation, reactor corrosion, poor catalyst recyclability and the need for treatment of waste effluent (Salem et al., 2013). Extreme pH can also affect the yeast or other microorganism’s performance during the fermentation process. Overall, enzymatic hydrolysis is safer and environmentally friendly. Hydrolysate containing sugars can be used for production of biofuel such as ethanol and butanol, or biochemicals such as succinic acid, acetic acid, lactic acid, levulinic acid, furfurals, biopolymers like polyhydroxybutyrate (PHB), polyhydroxyalkanoate (PHA), poly glutamate, etc. Canadian pulp and paper is the most energy-intensive industrial sector and one of the important components of the Canadian economy (CIPEC, 2008) and utilization of waste sludge to value added products will likely boost the country’s economy.

The merits of enzymatic hydrolysis over acid hydrolysis are: less inhibitor formation, undesirable by-products, reduction in processing waste generation, less expensive processing equipment (Dadi et al., 2006) and complete conversion efficiency (Wyman et al., 2005). Various acid hydrolysed biomasses showed glucose recovery but few glucose molecules are converted to
5-hydroxymethylfurfural (HMF) (Binder and Raines, 2010), a potent inhibitor of microbial fermentation (Klinke et al., 2004). Enzymatic hydrolysis of biomass is catalysed by a cellulase containing a mixture of endoglucanase, exoglucanase (cellobiohydrolase), and β-glucosidase. Endoglucanase break the noncovalent interactions present in the amorphous structure of cellulose, then exocellulase hydrolysis of chain ends to break the cellulose polymer into cellobiose and cellotriose followed by beta-glucosidase hydrolysis of disaccharides and tetrasaccharides into glucose (Horn et al., 2012). Many researchers studied the effect of different levels of all these 3 enzymes for enhanced cellulose or lignocellulose hydrolysis. Non-productive adsorption of enzyme onto lignin during biomass hydrolysis was efficiently reduced by some additives such as non-ionic surfactants or polymers. Principally, additives adsorb on lignin and prevent cellulase adsorption on lignin, ultimately cellulase adsorbs on cellulose for increased hydrolysis yields. Park et al. (1992) scrutinized the effect of several surfactants on enzymatic hydrolysis of newspaper, and double the conversion at 80h using Tween surfactant. Similar increased enzymatic hydrolysis yields were observations with Tween 80 reduced non-productive binding of enzyme on biomass surface (Eriksson et al. 2002). But, incomplete finding has been described about the encouragement of additives on hydrolytic capacities of cellulase desorbed from lignin and lignocellulosic materials.

The present study focuses on the enzymatic hydrolysis of pulp mill PS. The objective was to identify a method for and optimize the hydrolysis of PS to yield fermentable sugars. The impact of enzyme loadings, surfactant, and hydrolysis time on different PS solid loadings were also studied.
2.3. Materials and methods

2.3.1. Substrates, enzymes and surfactants

Primary sludge (PS) was provided by Terrace Bay Mill Inc. (Terrace Bay, ON, Canada). Enzyme used for enzymatic hydrolysis of PS was carried out with a commercial cellulase preparation Cellic® CTec2 (Novozymes A/S, Bagsvaerd, Denmark). Surfactants such as tween 20, tween 80, triton X 100 were purchased from Sigma Aldrich (MO, USA) and polyethylene glycol(PEG) 300, PEG4000, PEG 8000, PEG 10000 and PEG 20000 were purchased from Fisher Scientifics (NJ, USA).

2.3.2. Sludge and sugar analysis

The organic composition of PS was determined according to procedures TAPPI Test Methods (1984). An automated moisture analyzer (Sartorius MA37-1, Gottingen, Germany) was used to measure the total solid content of PS. To determine the sugar composition, 0.3 g of dried PS (DPS) sample was weighed in a clean screw capped test tube, and to that, 3 mL of 72% w/v H₂SO₄ was added. The well-mixed sample was hydrolyzed at 30 °C for 2 h with intermediate mixing. After 2 h of initial hydrolysis, 84 mL of deionized water was added, and sample was autoclaved at 121 °C for 1 h. Thereafter, the sample was cooled down to room temperature and filtered through a Whatman 0.45 µm filter paper. The solid residue collected on the filter paper was used for ash and acid insoluble (Klason) lignin determination. The supernatant was neutralized to pH 5.5–6.0 with CaCO₃ and filtered through a Whatman 0.2 µm filter paper prior to sugar analysis. For monomeric sugars analysis, samples with appropriate calibration standards were run on 1200 Series high performance liquid chromatography (Model 1260 Infinity, Agilent Technology, Toronto, ON, Canada) employing an Aminex HPX-87P column (Bio-Rad)
Laboratories, Hercules, CA, USA). Samples were processed at an eluent of HPLC grade deionized water with flow rate of 0.6 mL min$^{-1}$ using refractive index (RI) detector (Model G1362A, Agilent Technology) and The chromatograms were recorded and processed with an Open Lab CDS software (Agilent Technology). Calculations for cellulose (glucan) and hemicellulose (xylan, arabinan, mannan, galactan) were determined according to Alhammad et al. (2018) and Gao et al. (2014), respectively.

2.3.3. **Enzyme assay**

Cellulase activity of Cellic CTec2 enzyme was determined using a method as described by Adney and Baker (1996). The activities were measured spectrophotometrically and expressed as filter paper unit (FPU) per ml or g of enzyme. For recovered enzyme from hydrolysate, the cellulase activities were measured as stated earlier but final glucose analysis was done by HPLC method.

2.3.4. **Enzymatic hydrolysis**

Enzymatic hydrolysis of PS was carried out with a commercial cellulase preparation Cellic(®) CTec2 (Novozymes A/S, Bagsvaerd, Denmark). Enzyme used according to the manufacturer recommendations under optimum pH of 5.0 and temperature of 50°C. Each sample were placed in a shaking incubator (Innova 44, Maine, USA) at 200 rpm according to Alhammad et al. (2018).

2.3.5. **Effect of surfactant on enzymatic hydrolysis**

Enzymatic hydrolysis of PS with a cellulase CTec2 was carried out according to the manufacturer recommendations under optimum pH of 5.0 and temperature of 50 °C. The cellulase activity of the CTec2 was 120 FPU mL$^{-1}$ enzyme or 100 FPU g$^{-1}$ enzyme, based on enzyme density of 1.2 g mL$^{-1}$. Hence, to apply a 2% (w/w) enzyme loading (which corresponds to
12 FPU mL$^{-1}$ enzyme or 10 FPU g$^{-1}$ enzyme), 120 µL of enzyme was added in the experiments with 5% dry solids. The impact of surfactants (tween 20, tween 80, triton X 100, PEG 300, PEG 4000, PEG 8000, PEG 10000 and PEG 20000) on enzymatic hydrolysis was studied as described by Alhammad et al. (2018). Following pH and consistency adjustment, surfactants were mixed with PS at 1% concentrations (w/w) 24 h before addition of CTec2 cellulase. The pulp consistency, enzyme, surfactant and buffer were adjusted to 100%. To facilitate mixing during enzymatic hydrolysis, 10 g ash-free glass beads of size 210–300 µm (Sigma Aldrich) were added to each flask (250 mL) and samples were placed in a shaking incubator (Innova 44, New Brunswick, Canada) at 200 rpm. All samples were hydrolyzed in triplicates.

2.3.6. Experimental design and statistical analysis

Four parameters, solid loadings ($X_1$), enzyme loadings ($X_2$), surfactant, PEG 4000 loadings ($X_3$), and hydrolysis time ($X_4$) were selected as the most important independent factors based on literature reports and preliminary experiments (Das et al., 2015; Phummala et al., 2015). Optimization of enzymatic hydrolysis of PS was carried out by response surface methodological, central composite design (CCD) with four variables at five levels using Design Expert-8 software (Version 8.03, State-Ease, MN, USA). The parameters studied for enzymatic hydrolysis were: solid loading (5-15%, w/w), enzyme loadings (1-5%), surfactant- PEG 4000 concentration (1-5%) and hydrolysis time (48-144h). RSM with 30 experimental run containing 16-factorial, 8-axial and 6-central points were employed to study responses regarding glucose recoveries. The results of the experiments were used to determine the optimum combination of the variables for the best hydrolysis conditions. The results obtained through this experimental set up were fed to the software and analysed based on RSM. A polynomial quadratic regression equation was obtained which represents the effect of independent factors and its interactions towards the
glucose recoveries (% w/w dry PS). The interactive effects of parameters were analysed based on three dimensional response surface plots. The second-degree polynomials were calculated with Design-Expert software to estimate the response of the dependent variables (Eq. 1).

\[
Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i X_i + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j
\]  

(1)

where \( Y \) is the predicted response for glucose yield in %, \( \beta_0 \) is the intercept, \( b_i \) is the coefficient for linear direct effect, \( \beta_{ij} \) is the coefficient for interaction effect; \( \beta_{ii} \) is the coefficient for quadratic effect (a positive or negative significant value implies possible interaction between the medium constituents); \( X_i, X_{ij}, \) and \( X_{ii} \) are the independent variables. A quadratic polynomial equation was projected to describe the mathematical relationship between the response and the variables. The fit of the model was evaluated by the determination of \( R^2 \), adjusted \( R^2 \) coefficient and its statistical significance was determined by the \( F \)-test. The significant levels of the factors in the model were determined using analysis of variance (ANOVA) for each response. The effect of each independent variable and their interaction effects were determined. The number of parameters that were chosen to be included for each model were determined based on the significance (\( \alpha = 0.05 \)) of each model parameter using the \( F \)-test. To maximize the glucose recovery from PS, numerical optimization was used for determination of the optimal levels of the four variables.

2.3.7. Model validation

The optimizer predicts the optimized condition along with the predicted output. The validation experiment of enzymatic hydrolysis was carried out based upon the optimizer predicted output as described in “Results and Discussion”: DPS solids loading of 7.4%, enzyme loadings of 2.6% (19.2 FPU g\(^{-1}\)DPS, PEG 4000 loadings 5% and hydrolysis time of 100 h. All experiments
were performed in triplicate and standard deviations were calculated from the mean of the duplicate analyses.

2.4. Results and discussion

2.4.1. Compositional analysis

The compositional analysis of PS is presented as % of total dry weight of sludge in Table 2.1. The PS showed 70% moisture and 30% solids. These 30% solids constituted 61.6% polysaccharides (cellulose and hemicellulose), 23.5% Miscellaneous (proteins&extractives). The lignin and ash content of PS were 11% and 3.9%, respectively. Similar to this, Kang et al. (2010) analyzed kraft paper mill primary sludge and found 44.5% w/w glucan, 9.9% xylan and 8.1% lignin which suggest that the sludge biomass is rich source of carbohydrate, in particular cellulose content of sludge biomass. Thus, the high sugar content, of which majority was glucose, suggests that PS is a suitable feedstock for biorefinery applications (Alhammad et al., 2018).

2.4.2. Effect of surfactant

Surfactant showed positive effect on enzymatic hydrolysis of DPS to released maximum glucose recoveries. Among the 8 surfactants, enzymatic hydrolysis of DPS supplemented with PEG 4000 showed maximum 44.9% glucose recovery which was 14.9% accelerated when compared to control. PEG 300 and tween 80 also showed 38 and 41.1% glucose recoveries, respectively but, all other surfactant promoted the enzymatic hydrolysis for higher glucose recoveries in between 44.7–44.9%. Thus, surfactant enhances the enzymatic hydrolysis of DPS. Similar studies with PEG 4000 was conducted by Zhang et al. (2011) and concluded interaction of lignin and surfactant and thus unproductive binding of cellulase to lignin was inhibited which
resulted into increased cellulase activity. Literature survey showed different mechanism of action of surfactant during enzyme hydrolysis such as surfactant could change the nature of substrate (Zhang et al. 2009); surfactant could increase the stability of the enzymes (Gunjikar et al., 2001) and adsorption of surfactant to lignin surface could prevent non-specific binding between enzyme and lignin (Siposa et al., 2010). Overall, surfactant inhibited the lignin component and enhanced the cellulase hydrolysis.

**Table 2.1.**

Compositional analysis of primary sludge.

<table>
<thead>
<tr>
<th>Components</th>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>70.0</td>
</tr>
<tr>
<td>Solids</td>
<td>30.0</td>
</tr>
<tr>
<td>Cellulose (Glucan)</td>
<td>51.0</td>
</tr>
<tr>
<td>Hemicelluloses</td>
<td>10.6</td>
</tr>
<tr>
<td>Lignin</td>
<td>11.0</td>
</tr>
<tr>
<td>Ash</td>
<td>3.9</td>
</tr>
<tr>
<td>Miscellaneous (proteins &amp; extractives)</td>
<td>23.5</td>
</tr>
</tbody>
</table>
Fig. 2.2. Effect of surfactant on enzymatic hydrolysis of primary sludge [Solids loadings- 5% DPS, Enzyme loadings – 2% on DPS, Surfactant loadings -1%, pH 5 (50mM Sodium citrate buffer), Incubation temperature- 50°C, Agitation- 200 rpm].

2.4.3. Response surface methodology

The statistical combination of the test variables along with the measured response values, which were expressed as glucose recoveries, as summarized in Table 2.2. The application of the RSM yielded a regression equation, which was an empirical relationship between the glucose recovery and the test variables in coded units. The overall second-order polynomial equation for the enzymatic hydrolysis of DPS was as follows in Eq. 2:
Table 2.2.

Central composite design with observed and predicted response of glucose recoveries from DPS.

<table>
<thead>
<tr>
<th>Std. Run</th>
<th>Point Type</th>
<th>Solid loading (%)</th>
<th>Enzyme loading (%)</th>
<th>PEG 4000 loading (%)</th>
<th>Hydrolysis time (h)</th>
<th>Glucose recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Actual</td>
<td>Actual (coded)</td>
<td>Actual (coded)</td>
<td>Actual (coded)</td>
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<td></td>
</tr>
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<td>1 (-1)</td>
<td>48 (-1)</td>
<td>43.1</td>
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<td>1 (-1)</td>
<td>48 (-1)</td>
<td>12.7</td>
</tr>
<tr>
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<td>Factorial</td>
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<td>5 (+1)</td>
<td>1 (-1)</td>
<td>48 (-1)</td>
<td>35.8</td>
</tr>
<tr>
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<td>Factorial</td>
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<td>5 (+1)</td>
<td>1 (-1)</td>
<td>48 (-1)</td>
<td>08.0</td>
</tr>
<tr>
<td>5</td>
<td>Factorial</td>
<td>5 (-1)</td>
<td>1 (-1)</td>
<td>5 (+1)</td>
<td>48 (-1)</td>
<td>88.3</td>
</tr>
<tr>
<td>6</td>
<td>Factorial</td>
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<td>1 (-1)</td>
<td>5 (+1)</td>
<td>48 (-1)</td>
<td>23.2</td>
</tr>
<tr>
<td>7</td>
<td>Factorial</td>
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<td>5 (+1)</td>
<td>5 (+1)</td>
<td>48 (-1)</td>
<td>81.6</td>
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<tr>
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<td>Factorial</td>
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<td>5 (+1)</td>
<td>5 (+1)</td>
<td>48 (-1)</td>
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</tr>
<tr>
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<td>1 (-1)</td>
<td>1 (-1)</td>
<td>144 (+1)</td>
<td>63.9</td>
</tr>
<tr>
<td>10</td>
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<td>1 (-1)</td>
<td>1 (-1)</td>
<td>144 (+1)</td>
<td>09.9</td>
</tr>
<tr>
<td>11</td>
<td>Factorial</td>
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<td>5 (+1)</td>
<td>1 (-1)</td>
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</tr>
<tr>
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<td>1 (-1)</td>
<td>144 (+1)</td>
<td>05.5</td>
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<td>5 (+1)</td>
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<td>96.9</td>
</tr>
<tr>
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<td>5 (+1)</td>
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<tr>
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<td>3 (0)</td>
<td>96 (0)</td>
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<td>20</td>
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<td>10 (0)</td>
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<td>3 (0)</td>
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<td>21</td>
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<td>-1 (-2)</td>
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<tr>
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<td>7 (+2)</td>
<td>96 (0)</td>
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<tr>
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<tr>
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<td>3 (0)</td>
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</tr>
<tr>
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<td>Center</td>
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<td>26</td>
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<td>3 (0)</td>
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</tr>
<tr>
<td>28</td>
<td>Center</td>
<td>10 (0)</td>
<td>3 (0)</td>
<td>3 (0)</td>
<td>96 (0)</td>
<td>71.1</td>
</tr>
<tr>
<td>29</td>
<td>Center</td>
<td>10 (0)</td>
<td>3 (0)</td>
<td>3 (0)</td>
<td>96 (0)</td>
<td>74.7</td>
</tr>
<tr>
<td>30</td>
<td>Center</td>
<td>10 (0)</td>
<td>3 (0)</td>
<td>3 (0)</td>
<td>96 (0)</td>
<td>78.7</td>
</tr>
</tbody>
</table>
\[ Y_G = 77.41 - 16.32X_1 + 4.17X_2 + 10.20X_3 + 6.79X_4 + 2.58X_{12} - 13.45X_{13} - 2.10X_{14} + 3.72X_{23} - 1.90X_{24} + 2.37X_{34} - 15.98X_{11} - 18.26X_{22} + 0.14X_{33} - 7.97X_{44} \]  \tag{2}

In Eq. 2, \( X_1 \) is the solids loadings (\( \% \), \( \text{w w}^{-1} \)), \( X_2 \) is the enzyme loadings (\( \% \), \( \text{w g}^{-1} \) \( \text{DPS} \)), \( X_3 \) is the PEG 4000 loadings (\( \% \), \( \text{w w}^{-1} \) \( \text{DPS} \)), and \( X_4 \) is the hydrolysis time (h). The statistical significance of the model equation was checked with the \( F \)-test, and the analysis of variance (ANOVA) for the response surface quadratic model is shown in Table 2.3. The model \( F \)-value of 2.94 and the values of probability (\( P \) \( > F \) \( < 0.0001 \)) showed that the model terms were significant at 95\% confidence level (Zambare \textit{et al.} 2011). The coefficient of determination (\( R^2 \)), which was calculated for the glucose production, indicated that the statistical model explained 93\% of the variability in the response. Linear terms, two quadratic terms, and two interaction terms were significant. The coefficients of the response surface model are also presented in Eq. 2. Statistically, \( p \)-value greater than 0.05 indicates the terms were not significant. In this case, \( X_1 \), \( X_{11} \) and \( X_{22} \) were the significant model terms. The linear models for each response generated response surfaces. The response for the highest glucose recoveries was determined. According to Table 2.3, the \( R^2 \)-value was 0.93 in good agreement with the adjusted \( R^2 \)-value of 0.89. The vicinity of adjusted \( R^2 \) to \( R^2 \) means a good adjustment of the theoretical values to the experimental data by the model. So the adjusted model was suitable to predict the experimental data from enzymatic hydrolysis of DPS. The stronger model significant was confirmed by \( R^2 \) value as close as 1 (Gunawan \textit{et al.} 2005; Fang \textit{et al.}, 2010). The adequate precision measured the signal-to-noise ratio. Ratios greater than 4 indicated adequate model discrimination. The adequate precision of the developed model was 6.3 (Table 2.3) indicating that the model could be used to navigate the design space.
Fig. 2.3. Response surfaces plots representing the effect of four variables on glucose recoveries from DPS hydrolysate representing the effect of solids and enzyme loadings (a), solids loadings
and PEG 4000 loadings (b), solid loading and hydrolysis time (c), enzyme loadings and PEG 4000 (d), enzyme loadings and hydrolysis time (e) and PEG 4000 loadings and hydrolysis time (f).

**Table 2.3.**

Analysis of variance (ANOVA) of glucose recoveries from primary sludge as function of solids loadings ($X_1$), enzyme loadings ($X_2$), PEG 4000 loadings ($X_3$) and hydrolysis time ($X_4$).

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>DF</th>
<th>Mean square</th>
<th>$F$-value</th>
<th>$p$-value (Prob &gt; $F$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>29071.10</td>
<td>14</td>
<td>2076.5</td>
<td>2.94</td>
<td>0.0232*</td>
</tr>
<tr>
<td>$X_1$-Solid loadings</td>
<td>6395.75</td>
<td>1</td>
<td>6395.75</td>
<td>9.06</td>
<td>0.0088*</td>
</tr>
<tr>
<td>$X_2$-Enzyme loadings</td>
<td>417.56</td>
<td>1</td>
<td>417.56</td>
<td>0.59</td>
<td>0.4537</td>
</tr>
<tr>
<td>$X_3$-PEG 4000 loadings</td>
<td>2497.84</td>
<td>1</td>
<td>2497.84</td>
<td>3.54</td>
<td>0.0795</td>
</tr>
<tr>
<td>$X_4$-Hydrolysis time</td>
<td>1105.34</td>
<td>1</td>
<td>1105.34</td>
<td>1.57</td>
<td>0.2299</td>
</tr>
<tr>
<td>$X_{12}$</td>
<td>106.67</td>
<td>1</td>
<td>106.67</td>
<td>0.15</td>
<td>0.7029</td>
</tr>
<tr>
<td>$X_{13}$</td>
<td>2895.60</td>
<td>1</td>
<td>2895.60</td>
<td>4.10</td>
<td>0.061</td>
</tr>
<tr>
<td>$X_{14}$</td>
<td>70.63</td>
<td>1</td>
<td>70.63</td>
<td>0.10</td>
<td>0.7561</td>
</tr>
<tr>
<td>$X_{23}$</td>
<td>221.28</td>
<td>1</td>
<td>221.28</td>
<td>0.31</td>
<td>0.5837</td>
</tr>
<tr>
<td>$X_{24}$</td>
<td>57.80</td>
<td>1</td>
<td>57.80</td>
<td>0.08</td>
<td>0.7786</td>
</tr>
<tr>
<td>$X_{34}$</td>
<td>90.22</td>
<td>1</td>
<td>90.22</td>
<td>0.13</td>
<td>0.7256</td>
</tr>
<tr>
<td>$X_{11}$</td>
<td>7002.96</td>
<td>1</td>
<td>7002.96</td>
<td>9.93</td>
<td>0.0066*</td>
</tr>
<tr>
<td>$X_{22}$</td>
<td>9147.44</td>
<td>1</td>
<td>9147.44</td>
<td>12.96</td>
<td>0.0026*</td>
</tr>
<tr>
<td>$X_{33}$</td>
<td>0.53</td>
<td>1</td>
<td>0.53</td>
<td>7.56E-04</td>
<td>0.9784</td>
</tr>
</tbody>
</table>
2.4.4. Optimum level production

Model validation of optimum results showed 85.6% of glucose recovery at 100h of hydrolysis time which is 14.4% less than the predicted value which might be due to substrate availability or process deviation showed during the experimental set up. Considering the rate of glucose recoveries, 24 h hydrolysis time is an ideal with 51.8% glucose recovery but afterwards the rate was reduced to 85.6% at 100h (Fig 2.4). Hence even with not much of a difference between the predictive glucose recoveries by RSM model has more precision in predicting the optimum condition. Our earlier studies on RSM model validation of enzymatic hydrolysis of corn stover resulted into 57.6% actual glucose recovery which was in a fairly good agreement with the predicted value of 61.0% with solid loadings of 10% and enzyme loadings of 20 FPU g\textsuperscript{−1} DM (Zambare and Christopher, 2012)

![Optimum level enzymatic hydrolysis of DPS.](image)
References:


Lynd, L.R., Cushman JH, Nichols R.J., Wyman, C.E. Fuel ethanol from cellulosic biomass, Science, 251 (1991), 1318


Alhammad, Alaa, 2017. Increased Production of Cellulosic Sugar from Lignocellulosic Materials.


CHAPTER 3: ENZYME RECOVERY AND RECYCLING IN PRIMARY SLUDGE
CONVERSION FOR MAXIMUM GLUCOSE RECOVERY

3.1. Abstract

Cellulase can be effectively recovered from hydrolyzed sludge using an ultrafiltration recovery method. Cellulase enzyme recovery of 34.7% was obtained through 3 kDa protein cut-off Vivaflow 50 ultrafiltration membrane. Economic analysis shows that cost savings gained by enzyme recycling are sensitive to enzyme pricing and loading. At the demonstrated recovery and current loading of 2.6% (19.2FPU g\(^{-1}\) dry primary sludge). The recovered enzyme was reused on fresh sludge sample to produce a glucose yield of 82.3% (without PEG4000 surfactant). Present work suggests that primary sludge can add value in the form of fermentable sugars; and the opportunity to reuse close to 30% of the enzyme will significantly reduce the production costs of fermentable sugars such as glucose. Thus, enzymatic hydrolysis of paper mill sludge offers a cleaner and eco-friendly process for conversion of sludge to fermentable sugar and enzyme recycling, respectively.

**Keywords:** Enzyme recovery, Enzyme recycling, Ultrafiltration, Cellulase, Primary sludge, Glucose recoveries
3.2. Introduction

Enzyme cost is one of the primary expenses in a biomass-to-ethanol process. Leading enzyme development companies are focusing efforts on reduction of enzyme-manufacturing costs; however, even at estimated cost reduction levels (10- to 12-fold reduction of current cost of $5/gal of ethanol), enzymes still represent approx. $0.40–$0.50/gal of ethanol produced (NRC, 2014; US Dept. of Energy, 2014).

Several investigators have studied the technical feasibility of enzyme recovery and recycling (Gregg and Saddler, 1996; Moniruzzaman et al., 1997) during biomass hydrolysis. The practicability of utilization of paper sludge as a potentially attractive substrate requires the conversion of all of its cellulosic and hemicellulosic components to fermentable sugars, which could be further converted to fuels, biochemicals and biomaterials, such as bioethanol, biodiesel, organic acids and biodegradable plastics (Ohara, 2003; Morques et al., 2008). But with the depletion of global petroleum and its increasing prices, bioethanol and biodiesel has been becoming one of the most promising biofuel for global fuel market (Liang and Jiang, 2013). For biofuel or biochemical production, enzymatic hydrolysis is a substrate specific process which required very less energy, no inhibitors are produced because the bioconversion process occurs at milder condition without any chemical usage (Mendes et al., 2014). An ideal enzymatic hydrolysis process is expected to yield maximum conversion of substrate to sugars at high consistency within reasonable time with least enzymatic input. Enzymatic hydrolysis is affected by several factors such as substrate accessibility, substrate consistency, substrate loadings, enzyme loadings, inhibitors adsorptions, surfactant, and the effect of degree of synergy between various enzyme components in the cellulase enzyme mixture (Dyk and Pletschke, 2012). Binding of cellulase to
the biomass could lead to loss of enzyme during hydrolysis and filtration. Therefore, we examined the effect of adding low concentrations of a non-ionic surfactant to the hydrolysis, a process that was shown to enhance recovery of enzyme and increase yield by other researchers (Alkasrawi et al. 2003; Eriksson et al. 2002). This is believed to be owing to the surfactant reducing the nonspecific binding of the enzyme.

Pulp and paper mill industries are always associated with disposal problem of highly contaminated sludge or bio-solids. In countries with large scale pulp and paper production such as the United States and Canada, the huge amount of waste generated has prompted the government and industries to find new use of these bio-solids. Canadian paper mills are taking initiative in minimizing the waste stream and converting it into a value-added product that can diversify their product range and markets, create additional revenues, and enhance their competitiveness. The present study evaluates methods to recover and recycle enzymes for hydrolysis of primary sludge and determine the economic contribution of recovery and recycle to overall process economics in a negative value sludge biomass-to-fermentable sugar process.

3.3. Materials and methods

3.3.1. Substrates, enzymes and surfactants

Primary sludge (PS) was provided by Terrace Bay Mill Inc. (Terrace Bay, ON, Canada). Enzyme used for enzymatic hydrolysis of PS was commercial cellulase preparation Cellic® CTec2 (Novozymes A/S, Bagsvaerd, Denmark). Total eight surfactants such as tween 20, tween 80, triton X 100 were purchased from Sigma Aldrich (MO, USA) and polyethylene glycol(PEG) 300,
PEG4000, PEG 8000, PEG 10000 and PEG 20000 were purchased from Fisher Scientifics (NJ, USA).

3.3.2. *Enzymatic hydrolysis*

Enzymatic hydrolysis of PS was carried out with a working volume of 100 ml containing dry PS solids loading of 7.4 wt.%, enzyme loadings with 50mM citrate buffer were autoclaved of at 121 C for 60 min. PEG-4000 (5 wt.%) was added 24 h before addition of enzyme. After 24 h, cellulose enzyme 2.6% (19.2 FPU g⁻¹DPS) was added and incubated at 50 C and 200 rpm. Samples were withdrawn after every 24 h up to 100 h. All experiments were performed in triplicate and standard deviations were calculated from the mean of the duplicate analyses.

3.3.3. **Enzyme assay**

Cellulase activity of CelliC CTec2 enzyme was determined using a method as described by Adney and Baker (1996). The enzyme assay for recovered enzyme was conducted using filter paper assay method as described in Chapter 2 but its glucose analysis was done by using HPLC method.

3.3.4. **Enzyme recovery**

The methodology for cellulase enzyme recovery and recycling from PS hydrolysate was represented in Fig 3.1.
The enzyme hydrolysate (100 mL) obtained from the optimum level procedure described in the previous section were centrifuged using Sorval RT1 centrifuge (Thermo Scientific, USA) at 10,000 rpm for 10 min. The particle free supernatant then ultra-filtered using a Vivaflow® 50 disposable and ready-to-use crossflow cassette (Sartorius, Gottingen Germany), containing a 50 cm² polyethersulfone ultrafiltration membrane with a molecular weight cut-off of 3 kDa. Before
ultrafiltration of the hydrolysate solutions, the ultrafilter was prewashed with deionized water followed by citrate buffer. The ultrafiltration was performed at 1–1.2 bar pressure until the desired reduction in volume of the retentate was achieved. Samples of the feed, retentate, and permeate solutions were analyzed for cellulase activity, and sugars content. After ultrafiltration the cartridge was washed with buffer to recover enzyme present in the solution that remained inside the ultrafilter and in the lines. The set-up of enzyme recovery using ultrafiltration is shown in Fig. 3.2.

![Fig. 3.2. Enzyme recovery set up using ultrafiltration method.](image)

### 3.3.5. Enzyme recycling

Enzyme recycling was done with 30% recovered enzyme with fresh 70% enzyme on 7.4% DPS supplemented with 1, 2.5 and 5% (w w⁻¹DPS) of PEG 4000. A positive control of 100% fresh enzyme and a negative control of combination of recovered and fresh enzyme with 5% PEG 4000 were used. Enzyme hydrolysis under optimum hydrolysis conditions (pH 5 and 50°C, and 200
rpm) for maximum glucose recovery was studied up to 100 h. Samples were analyzed for glucose using HPLC method as described earlier.

3.4. Results and discussion

3.4.1. Enzymatic hydrolysis

Enzymatic hydrolysis of PS under optimal hydrolysis condition showed increased glucose recoveries over time, with maximum glucose recoveries of 85.6% at 100h of hydrolysis time (Fig 3.3).

![Glucose Recovery vs Time Graph](image)

**Fig. 3.3.** Enzymatic hydrolysis of PS.

3.4.2. Enzyme recovery

Table 4 shows cellulase recovery from PS hydrolysate though different successive steps of centrifugation, filtration and ultrafiltration. Wang *et al.* (2012) reported various cellulase enzyme recyclability options regards to substrate when studying the enzymatic saccharification for biofuel
production. Initial cellulase activity of 22.8 FPU fed for hydrolysis of PS which was reduced down to 10.6 FPU in hydrolysate filtrate. This indicated that the filtrate still contains 46.9% cellulase enzyme. This suggests that most of the enzyme remains with the sludge and recovery is not feasible or part of some enzyme get deactivated during the hydrolysis process (Jørgensen and Pinelo, 2017). Guerfali et al. (2015) studied a cellulase enzymatic mixture and Kamaistone K-050 cellulase for hydrolysis of waste paper and found better functional stability with retention of about 66 and 71% of their initial activities, respectively. This might be due to their good thermal stabilities. Thus, operational stability is a key factor of enzymatic characteristic for industrial applications especially in continuous hydrolysis process and subsequently can reduce significantly the cost of lignocellulosic conversion.

Ultra-filtration of filtrate showed total 34.7% cellulase recovery including 29.6% and 5.1% from retentate and membrane washout, respectively. No cellulase was found in permeate (Table 3.1). Similar type of enzyme recovery of Cellic® CTec3 enzyme using ultrafiltration method was done from municipal solid waste (MSW) paper pulp (Puri et al., 2013). It has been observed that maximum 30% enzyme recovery at pH 9 (alkaline condition). From MSW paper pulp, only 15% enzyme was recovered at pH 5 however, our results showed relatively more than 2-fold higher enzyme recovery compared with the available literature of Puri et al. (2013). As the enzymes primarily remain bound to the sludge, these results indicated that it might be more advantageous to recycle the unhydrolysed sludge with its bound enzymes in addition to enzyme recovery from hydrolysate (Xue et al., 20102).
Table 3.1.

Enzyme recovery from sludge hydrolysate

<table>
<thead>
<tr>
<th>Recovery Factors</th>
<th>Volume (mL)</th>
<th>Total activity (FPU)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Enzyme Feed</td>
<td>100</td>
<td>22.80</td>
<td>100</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>57</td>
<td>10.60</td>
<td>46.91</td>
</tr>
<tr>
<td>Ultrafiltration- Concentrate</td>
<td>15</td>
<td>6.75</td>
<td>29.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>34.70</td>
</tr>
<tr>
<td>Ultrafiltration- Washout</td>
<td>28.50</td>
<td>1.16</td>
<td>5.09</td>
</tr>
<tr>
<td>Ultrafiltration- Permeate</td>
<td>35.50</td>
<td>0.00</td>
<td>0</td>
</tr>
</tbody>
</table>

3.4.3. Enzyme recycling

Recycling of recovered enzyme 30% (6.8 FPU) along with fresh enzyme 70% (16 FPU) on fresh 7.4% dry PS with 5% PEG4000 supplementation showed 78.5% glucose recovery after 100 h of hydrolysis (Fig. 3.4). This result of glucose recovery showed as closed as 80.7% of fresh and new enzyme (22.8 FPU) using 5% PEG 4000 at same hydrolysis conditions and time. The hydrolysis and glucose recovery trend was similar to model validation results, indicating the 100% performance of recovered enzyme on fresh DPS hydrolysis for released the optimum glucose recovery. Gomes et al. (2016) also studied recycling of cellulase from recycled paper sludge hydrolysate as well as unhydrolyzed sludge with 70-80% cellulase recycling up to 4 cycles. But after 1st recycling, the present study on glucose recovery and Gomes et al. (2016) reported glucan
conversion to be very close to 82.3 and 86%, respectively. Thus, the present study needs to improve the dose of recovered enzyme for recycling studies. Wang et al. (2016) reported cellulase recycling on hardwood Kraft based dissolving pulp utilizing 48.8–35.1% of recovered enzyme from filtered liquor in 5 recycle round which can be reused for enzymatic treatment of dissolving pulp. Wang et al. (2016) studies concluded an effectiveness of cellulase recycling was found to be dependent on alpha cellulose, alkali solubility and molecular weight distribution Thus, enzyme recycling is directly related to the cost-effectiveness of any industrial process. It was also observed that this process needs an excess PEG perhaps by acting as enzyme stabilizer (Li et al. 2012) (Eckard et al., 2013). By recycling and reusing cellulase, the enzyme treatment showed great potential towards industrial application.

![Graph](image)

**Fig. 3.4.** Partial (30%) substitution of fresh enzyme with UF-recovered enzyme during enzymatic hydrolysis of PS in presence of PEG4000 [D - 30% Recovered Enzyme +70% New Enzyme +5% PEG4000; E - 100% New Enzyme +5% PEG4000].
References


CHAPTER 4: CONCLUSIONS AND FUTURE WORK

The objective of this work was to evaluate technologies to effectively convert biomass sources for sustainable production of fermentable sugars (glucose). Lignocellulosic biomass is the only renewable source on earth that can sustainably produce biofuels and biochemicals without affecting the environment and food security, and replace fossil fuels and petrochemicals currently produced from oil. The development of cost-efficient bioconversion technologies for biomass valorization to biofuels and value-added bioproducts would contribute to the effort of building the Forest Biorefineries as a major pillar for the emerging Bioeconomy.

The first chapter presents a comprehensive review of modern literature on the topic. Based on the latest literature on this subject, a major research trend has been on sugar production from plant biomass through enzymatic hydrolysis, biomass formation, methods of treatment, advantages and disadvantages, opportunities for enhancing sugar yields from biomass, making enzymatic hydrolyses more cost-effective. The review indicates that this research area still needs further development to overcome the challenges of the economies of operations that currently prevent the applications at large-scale plants.

The second chapter has focused on improving enzymatic hydrolysis step by controlling different factors: 1) surfactants; 2) biomass (substrate) consistency; 3) hydrolyses time; 4) enzyme loading; It was found that using PEG 4000 had an effect on enzymatic hydrolysis, which increase glucose recoveries by more than 12% during initial screening. Also, it was found that with using
response surface methodological optimization and model validation of enzymatic hydrolysis with 7.4% of solid loading, 2.6% of enzyme loading, 5% of surfactant loading, at 100 h of hydrolysis time the highest glucose recoveries was found which reached 85.6%. This result can reduce the cost of hydrolyses process especially with low loadings of enzyme. Thus, surfactants has played a major role to improve enzyme activity during enzymatic hydrolysis of lignocellulosic biomasses, however, its actual mechanism of action is still poorly understood. The surfactant effect might be due to reduction in liquid viscosity, enzyme adsorption to non-specific sites, biomass modifications and air liquid interface contact of enzyme. Overall, surfactants plays a major role to enhance cellulose conversion in terms of reduction in enzyme usage, time, production and operating cost.

The last chapter present results and approaches to reduce the cost of enzymatic hydrolyses process by recovery and reuse of the enzyme (cellulase), ultrafiltration method help to recover 34.7% of cellulase protein that present in the liquid phase, and that will help reduce 30% of enzyme cost by using surfactant to recycle the enzyme for bioconversion of biomass.

Finally, this study would offer researchers further research insights related to the subject of cost-effective production of fermented sugars from lignin biomass: 1) A better understanding of the mechanisms of different factors with biomass during enzymatic hydrolysis; 2) Improved enzymatic hydrolyses process; 3) Opportunity to recycle enzyme and improve enzyme recovery.